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(57) Abstract

Polyalkylene oxide (PAO)-based compositions containing isocyanate and/or isothiocyanate groups for covalent attachment to bio-effecting substances such as peptides or chemotherapeutics are disclosed. The compositions react readily with the bio-effecting substances to provide compositions having increased circulating lives in mammals, substantially reduced immunogenicity and enhanced aqueous solubility. Methods of preparing such PAO-based compositions are also disclosed.

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NOVEL ATTACHMENT OF POLYALKYLENE OXIDES TO BIO-EFFECTING SUBSTANCES

BACKGROUND OF THE INVENTION

The present invention relates to modifications of bio-effecting substances with polyalkylene oxides. In particular, the invention relates to modifications which extend the circulating life and reduce the immunogenicity of bio-effecting substances.

Some of the unique properties of polyalkylene oxides (PAO's) such as polyethylene glycol (PEG) to reduce the immunogenicity and antigenicity of therapeutic agents such as peptides have been set forth in U.S. Patent Number 4,179,337. The attachment of such non-immunogenic polymers has also been found to prolong the circulating life of several therapeutic products in the bloodstream when compared to unmodified or native material.

Polyethylene glycol has several advantageous properties. The toxicity of PEG's of molecular weights greater than 1,000 daltons is very low. Consequently, the polymer can be safely administered intravenously in a wide variety of mammals. The polymer also has a wide range of solubilities. Polyalkylene oxides are soluble in most organic solvents as well as aqueous solutions. Many polypeptides and other materials conjugated with PEG and PAO's not only retain their solubility properties, but also demonstrate enhanced water solubility as a result of the conjugation. In addition, the biological activities of PEG conjugates are typically dominated by the non-PEG part of the conjugated molecule.

In order to covalently attach PAO's to bio-effecting materials such as proteins, the hydroxyl end-groups of the polymers must first be converted or activated into

reactive functional groups. For example, the use of PEG-succinimidyl succinate (SS-PEG) as a conjugating agent has been suggested. The activated polymer reacts with proteins under mild conditions while preserving biological activities. The resultant ester linkage, however, has limited stability in aqueous media.

The formation of urethane (carbamate) linkages between the amino groups of a protein and PEG has provided one solution to the problem of hydrolytic release of the polymer chains. Attachment of PEG to a protein via a carbamate derivative was disclosed in Analytical Biochemistry 131, 25-33 (1983) using carbonyl diimidazole-activated PEG. The activated polymer, however, is not very reactive with proteins, often requiring two to three days to achieve sufficient modifications. Another drawback associated with carbonyl diimidazole is its high relative cost.

Commonly assigned U.S. Patent No. 5,122,614 provides further improvements in PAO's which form urethane linkages with proteins. For example, the patent discloses protein modification with succinimidyl carbonates of PEG (SC-PEG) and bifunctional derivatives thereof (BSC-PEG) carried out within short periods of time over a broad pH range. The use of succinimidyl carbonates of PEG, however, cannot be universally applied to all bio-effecting materials. For example, under certain circumstances, some rearrangement of the SC-PEG has been observed, especially at higher temperatures. Beta alanine, often an unwanted by-product, is produced as a result of this rearrangement. In addition, substantial pH adjustment is required to maintain optimum reaction speed and minimize rearrangement.

Indirect attachment of isocyanate-containing substituents to PEG for the purpose of modifying bio-

effecting agents has also been suggested. See, for example Eur.Polym.J. Vol.19, No.12, 1177-1183 (1983) and Eur.Polym.J. Vol.19, No.4, 341-346 (1983). The formation of PEG-$O_2CNH-(CH_2)_6-NCO$ or PEG-substituted- alkyl or aryl-isocyanate has been reported as a possible amine and/or alcohol group modifier. Such indirect polymer coupling technology, however, suffers from several significant shortcomings and drawbacks. For example, such PEG-diisocyanates are inefficiently prepared. Reacting PEG with the hexamethylene diisocyante is incomplete and yields mono- and diisocyanates while leaving significant amounts of unreacted products. In order to overcome this drawback, excess amounts of diisocyanates must be employed and then removed. Moreover, unlike technology using direct linkage of PEG to a target amine, the indirect attaching group which separates the PEG and amine is susceptible to breakdown into several undesirable byproducts, including potentially toxic aliphatics during in vivo metabolic processes.

Each of the activated forms of the PAO-based polymers described above has properties which, under certain circumstances, may be considered advantageous. Nevertheless, extending the PAO-modifying reagents and techniques to include additional proteins, peptides or chemotherapeutic agents which heretofore have not benefitted from such modifications because of unreactivity, harmful by-products and the like is desirable.

It would be advantageous to provide stable PEG-modified bio-effecting agents which maintain the activity of the functional moieties in the body for extended periods. It would also be beneficial to provide such PEG-conjugates in a form having substantially reduced immunogenicity, improved solubility and pH stability.

It is therefore, an object of the present invention to provide improved therapeutic agents using PAO modification techniques. Other and further objects of the invention will be apparent from the disclosure set forth herein.

SUMMARY OF THE INVENTION

In accordance with the present invention, improvements in polyalkylene oxide(PAO)-based therapeutics are disclosed. In one aspect of the invention, PAO - based compounds are disclosed having the formula:

(I) $Z - (O - R_1)_a - (O - R_2)_b - (O - R_3)_c - Y$

wherein:

R_1, R_2 and R_3 are alkyl groups;

a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b and c is between 10 and 1000;

Y is NCX, wherein X is one of O or S; and

Z is one of an alkyl, cycloalkyl, branched alkyl, arylalkyl group or Y.

In this aspect of the invention, each of R_1, R_2 and R_3 can be straight or branched alkyl groups and/or each of R_1, R_2, R_3 can be independently the same or different from each other. In a particularly preferred aspect of the invention, the PAO-based compositions are

polyethylene glycol-based, having a molecular weight of from about 2,000 to about 20,000.

In another aspect of the present invention, methods are provided for preparing the compositions of (I). The methods include reacting compounds having the formula:

(II) $A-(O-R_1)_a-(O-R_2)_b-(O-R_3)_c-NH_2$

wherein R_1, R_2 and R_3 are alkyl groups;
a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b and c is between 10 and 1000; and
A is one of an alkyl, cycloalkyl, branched alkyl, arylalkyl or amino group;
with phosgene or a phosgene-containing substance such as triphosgene in the presence of a suitable base.

A further aspect of the invention provides compositions containing bio-effecting substances covalently bound to at least one PAO-based derivative. The covalent linkage achieved through the isocyanate and/or isothiocyanate portion of the activated PAO-based polymer derivatives (I) and an alcohol present in the bio-effecting substance produce a urethane and/or thiourethane linkage. The covalent linkage between the reactants can also be achieved through the same isocyanate or isothiocyanate portions of the activated PAO-based polymers and amines present in the bio-

effecting substance to produce urea and/or thiourea linkages.

A still further aspect of the invention is directed to methods for preparing such bio-effecting-PAO compositions. The methods include reacting the PAO-based polymer with the bio-effecting substance under conditions which effect covalent attachment of the ingredients at the isocyato- and/or isothiocyato- portion of the polymer and amine and/or alcohol portion of the bio-effector. The conditions are further defined as those which permit the covalent attachment of the reactants while substantially maintaining the desired effect of the bio-effecting substance.

The applications of the present invention are vast. The activated PAO's of the present invention equip the artisan with a polymer-based material that is suitable both for amine-based and alcohol-based linkages. Indeed, it is now possible to attach the polymers to bio-effecting substances at both locations at will in order to maintain optimal activity of the native portion of the molecule. The ability to effect such mixed attachments of the reactants allows the artisan to precisely attach the beneficial polymer where desired and more precisely modulate the activity of the therapeutic conjugates.

As a result of the present invention, PAO-based polymers are provided which improve a wide range of

therapeutic substances. The conjugation prolongs the circulating life of the substances when compared to unmodified forms. Thus, it is possible to substantially reduce the amount and frequency of administrations without diminishing the therapeutic effect. Such reductions also reduce the concomitant toxicities often seen when high doses of agents are administered, especially over time. In addition, the novel covalent conjugation of PAO's to bio-effecting substances also improves the aqueous solubility of the bio-effector and allows the artisan to provide therapeutic compositions in heretofore impractical dosage forms.

The most dramatic of all improvements achieved as a result of the present invention is often the substantial reduction of the immunogenic and/or allergic responses of the compositions of the present invention when compared to the unmodified or native bio-effecting counterparts. Whereas in the past, such therapy-limiting consequences often would mean discontinuing treatment, the compositions of the present invention allow not only continued treatment but also substantial reductions in untoward reactions.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes activated polyalkylene oxides having the general structure:

(I) $Z - (O - R_1)_a - (O - R_2)_b - (O - R_3)_c - Y$

wherein:

R_1, R_2 and R_3 are alkyl groups;

a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b and c is between 10 and 1000;

Y is NCX, wherein X is one of O or S; and

Z is one of an alkyl, cycloalkyl, branched alkyl, arylalkyl group or Y.

An important feature of the activated PAO's described herein is the presence of Y in a terminal position. The NCO or isocyanate portion of the activated polymer reacts rapidly with nucleophiles such as the epsilon-amino group of lysine moieties or the hydroxyl or alcohol groups of various bio-effecting substances to produce stable, conjugated molecules.

The stability of the conjugate is achieved by covalent linkage between the isocyanate and/or isothiocyanate portion of the activated PAO-based polymer derivatives (I) and an alcohol present in the bio-

effecting substance to produce a urethane and/or thiourethane linkage. The covalent linkage between the reactants can also be achieved through the same isocyanate or isothiocyanate portions of the activated PAO-based polymers and amines present in the bio-effecting substance to produce urea and/or thiourea linkages. Conjugation with the isocyanate-activated PAO's is preferred.

The stability of the conjugates is superior to that of many other PEG conjugations which depend upon ester linkages, especially in the area of hydrolytic stability. In addition, conjugation reactions combining the novel activated PAO's described herein can be carried out with minimal or no pH adjustment to maintain an optimal reactivity range.

Within the compositions of (I), suitable alkyl groups include, for example, ethyl, propyl, isopropyl or butyl. Cycloalkyl groups include cyclohexyls, for example; suitable branched alkyls include materials such as 3-methylhexyl, for example and suitable arylalkyls groups include, for example, benzyl.

In particular, the present invention relates to PAO-based polymers which are water soluble and do not generate an immunogenic response when administered to mammals. The polymeric substances included herein are also preferably water-soluble at room temperature. A wide

variety of materials are thus suitable for use herein. A non-limiting list of such polymers include: polyalkylene oxide homopolymers such as polyethylene glycol or polypropylene gylcols, polyoxyethylenated polyols, copolymers thereof and block copolymers thereof, provided that water solubility of the block copolymer is maintained. In addition, each of R_1, R_2 and R_3 may be straight or branched alkyl groups. Alternatively, each of R_{1-3} may be independently the same as or different from the others of R_{1-3}. For example, each of R_{1-3} can be CH_2CH_2, CH_2CHCH_3 or $CH_2CH_2CH_2CH_2$.

Polyethylene glycols are the preferred polyalkylene oxides (PAO's) described herein. Although polyethylene glycols come in a variety of molecular weights, averaging from about 600 to about 100,000 daltons, the preferred molecular weight range for modifying therapeutic agents is from about 2,000 to about 20,000 daltons. Molecular weights of from about 3,000 to about 7,000 are especially preferred.

The molecular weight of the polymer will vary depending upon the needs of the artisan and the particular bio-effecting substance to be modified. Those of ordinary skill in the art can determine the molecular weight ranges suitable for particular end use applications.

The foregoing is merely illustrative not to be considered restrictive of the type of materials suitable for use herein. Those skilled in the art will realize that equivalent or substantially equivalent polymeric materials not specifically mentioned but having the qualities described herein are also contemplated.

In a particularly preferred aspect of the invention, new, activated polyethylene glycols are provided. Within this aspect are poly(ethylene glycol)-isocyanates (PEG-NCO) and their bifunctional derivatives, PEG bis-isocyanate, as well as poly(ethylene glycol)-isothiocyanates (PEG-NCS) and their bifunctional derivatives. Thus, for example, in (I), Z can be the same as Y, that is, Z = isocyanate or isothiocyanate. Heterobifunctional derivatives of PEG and PAO's are also possible. In this scenario, Z may be an alternative functional group to that of Y, that is, Z = isothiocyanate when Y = isocyanate. Alternative functional groups for Z such as hydrogen, an alkyl group, a chloroformate or an N-dicarboximide are also contemplated.

The activated PAO's of (I) are generally prepared by reacting compounds having the formula:

(II) $A-(O-R_1)_a-(O-R_2)_b-(O-R_3)_c-NH_2$

wherein R_1, R_2 and R_3 are alkyl groups;

a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b and c is between 10 and 1000; and

A is one of an alkyl, cycloalkyl, branched alkyl, arylalkyl or amino group;

with phosgene or a phosgene-generating substance in the presence of a suitable base. In the case of A = an amino, a bifunctional diisocyanate is formed. The reaction can be carried out over a broad temperature range of from about 10 to about 130° C.

In the case of the preferred PEG-based polymers, initially, methoxy PEG, mPEG, readily available from Aldrich Chemical of Milwaukee, WI is converted to mPEG-NH_2 by treatment with thionyl chloride to form mPEG-Cl followed by treatment with aqueous ammonia. See, for example, Biotech. App. Biochem. 9, 258-68 (1987). Thereafter, PEG isocyanate or PEG isothiocyanate formation is accomplished by reacting the PEG-amine with phosgene or thiophosgene or similar generating substances such as bis-(trichloromethyl)carbonate (triphosgene) in the presence of a base such as triethylamine. Suitable alternative bases include pyridine, N,N-dimethylaniline, 1,4-diazabicyclo[2.2.2]octane or DABCO™, available from Aldrich Chemical Co. of Milwaukee, WI. or any other suitable inert tertiary amine bases.

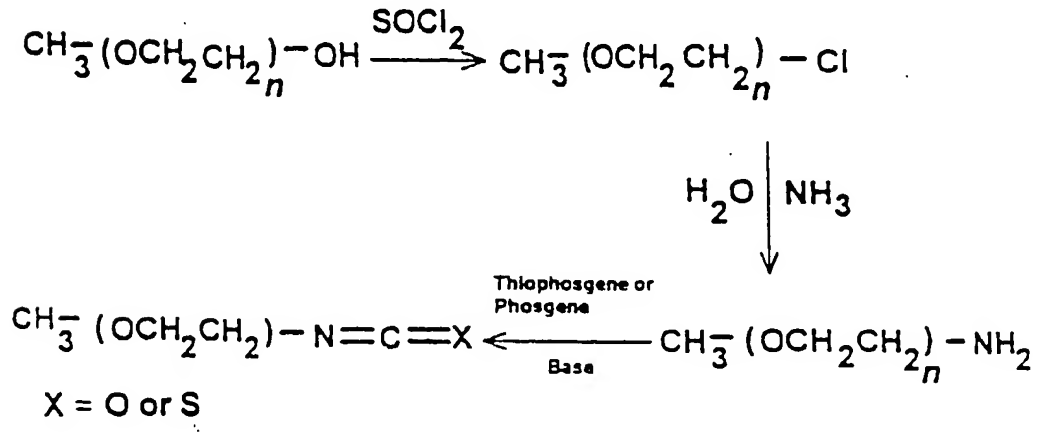
The amount of activated PAO required to modify and

conjugate with a subject bio-effecting molecule will depend on several factors. Principally, however, the amount of polymer is dependent upon the needs of the artisan and the degree of modification desired. The ratio of activated polymer to bio-effecting substance will accordingly vary widely. Molar ratios on the order of from about 0.1 : 1 to about 50 : 1 are contemplated, with ratios of from about 1 : 1 to about 25 : 1 being preferred and about 5 : 1 to about 20 : 1 being most preferred. Protein modification, for example, often requires a molar excess of the activated polymer. A 5 to 40-fold molar excess of PEG-NCO is sufficient to modify hemoglobin.

Activation of the PAO-based polymer with isocyanate is depicted below in Scheme 1, using PEG as the representative polymer:

SCHEME 1

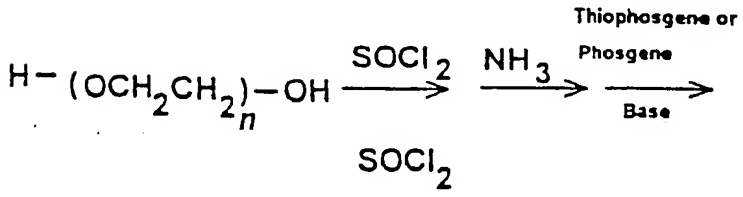
Synthesis of mPEG-NCO

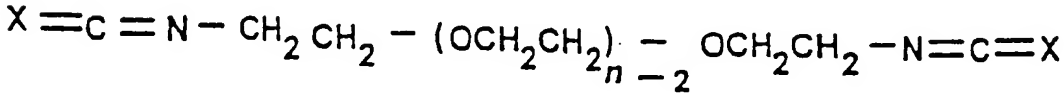


Activation of the polymer with isocyanate into the bifunctional derivative using PEG as the illustrative polymer is set forth below in Scheme 2:

SCHEME 2

Synthesis of PEG-bisNCO





X = O or S

The above-described activated PAO's can be covalently attached to a wide variety of bio-effecting substances to provide compositions having the general formula:

(III) $Z'-(O-R_1)_a-(O-R_2)_b-(O-R_3)_c-NH-CO-W-R''$

wherein R_1, R_2 and R_3 are alkyl groups;

a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b and c is between 10 and 1000;

W = O or NR, wherein R = H or an alkyl;

R" is a bio-effecting substance; and

Z' is an alkyl, cycloalkyl, branched alkyl, arylalkyl or -NH-CO-W-R"

As used herein, the term "bio-effecting" substance is to be broadly construed and means any substance displaying a physiologic effect preferably in mammals after administration, whether oral, parenteral or otherwise. Within this broad gamut of substances, there are included peptides, polypeptides and enzymes, both naturally and synthetically derived. The present invention also contemplates attachment of the activated PAO's to chemical moieties such as chemotherapeutic substances. A non-limiting list of such bio-effecting substances includes:

a) enzymes such as asparaginase, arginase, adenosine deaminase, superoxide dismutase, catalase, chymotrypsin, lipase, uricase, bilirubin oxidase, glucose oxidase, glucuronidase, galactosidase, glucocerebrosidase and glucuronidase;

b) polypeptides such as Factor VIII, insulin, ACTH, glucagon, somatostatin, somatotropins, thymosin, parathyroid hormone, pigmentary hormones, somatomedins, erythropoietin, luteinizing hormone, hypothalamic releasing factors, antidiuretic hormones, prolactin, interleukins, interferons and

colony stimulating factors, hemoglobin, cytokines and antibodies;

c) glycopolypeptides such as immunoglobulins, ovalbumin, lipase, glucocerebrosidase, lectins, tissue plasminogen activator and glycosilated interleukins, interferons and colony stimulating factors.

d) immunoglobulins such as IgG, IgE, IgM, IgA, IgD and fragments thereof.

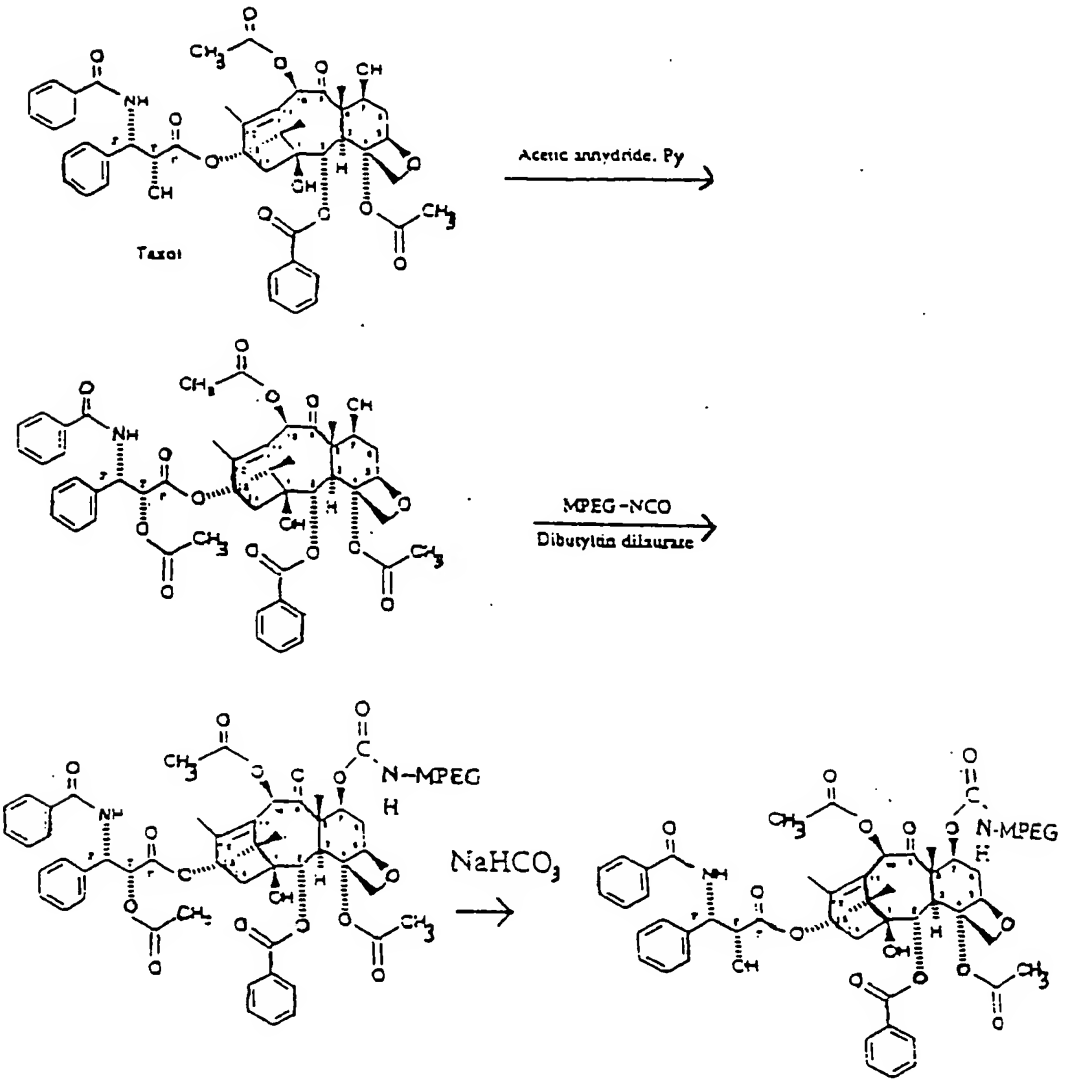
e) chemotherapeutic agents of all therapeutic categories such as anti-inflammatory agents, anti-infective compositions such as antibiotics, including hydroxy- or amino-penicillins or cephalosporins, anti-tumor agents such as taxol or taxol derivatives including 2'-acetyltaxol or methotrexate and the like, analgesics such as opiates, cardiac agents, steroids such as corticosteriods, central nervous system agents, etc.

The only limitation on the type of bio-effecting substances suitable for use herein is that the substance contain at least one amine or alcohol substituent for covalent attachment of the activated PAO without substantially reducing or eliminating the therapeutic effect of the unmodified material.

As depicted in Scheme 3 below, when attachment is effected between the isocyanate portion of the activated PEG and alcohols on the bio-effecting substance, stable urethane linkages are formed. Tin-based catalysts such as dibutyltin dilaurate are advantageously included in such reactions. Similarly, when isothiocyanate portions of the activated polymers are reacted with hydroxyls on the bio-effectors, thiourethane linkages are formed.

SCHEME 3

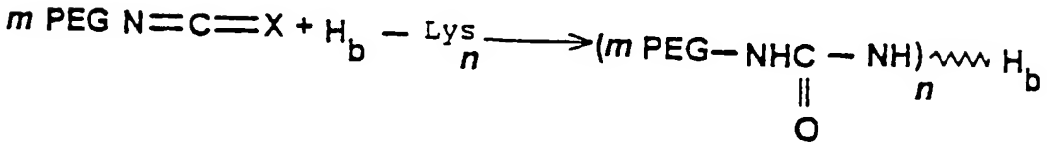
PEG-NCO MODIFICATION OF HYDROXYL-CONTAINING BIO-EFFECTING SUBSTANCE TAXOL



As shown in Scheme 4 below, when the isocyante portions of the activated PEG's are reacted with amines on the bio-effecting substance, stable urea linkages are formed.

SCHEME 4

PEG-NCO MODIFICATION OF AMINE-CONTAINING BIO-EFFECTING SUBSTANCE HEMOGLOBIN



The above-described activated PAO's afford substantial modification of a wide variety of bio-effecting substances. The modification reactions described above can be carried out in both aqueous and non-aqueous solvents. For example, protein modification is achievable under mild conditions using aqueous systems having a pH ranging from about 5.0 to about 11.0 and preferably from about 7.0 to about 8.0, moderate reaction temperatures of from about 0 to about 10^0 C., and reaction times of from about 0.25 to about 1 hour. In non-aqueous systems where hydroxyl moieties are linked to the polymers, more rigorous temperature and time conditions are sustainable and will generally include tin-based catalysts to speed up the reaction.

EXAMPLES

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention.

EXAMPLE 1

Preparation of mPEG-NH_2

mPEG-NH_2 was prepared using procedures similar to those described in Biotech. App. Biochem. 9, 258-268 (1987). In a 1 liter round bottom flask, 100 grams of mPEG-5000 was melted and then dried under high vacuum. Twenty grams of thionyl chloride was then added to the dried mPEG and the mixture was heated to 65-70 degrees C. for 5 hours using a reflux condenser. The unreacted thionyl chloride was removed with a rotary evaporator and high vacuum. A 2 gram sample of this melt was recrystallized from 2-propanol. The ^{13}C NMR spectrum ($CDCl_3$) showed a peak at 42 ppm corresponding to $\underline{C}H_2$-Cl. A peak at 61.5 ppm (corresponding to CH_2-OH) could not be detected indicating that the conversion was greater than 95%.

Thereafter, 150 mL of deionized water and 250 mL concentrated ammonia were added to a flask containing the mPEG and the flask was heated at 60°C for 4 days using

a reflux condenser. The reaction mixture was then stirred under aspirator vacuum at 60° C. for 4 hours to remove ammonia. The reaction mixture was then cooled to room temperature and to the flask were added 20 grams of K_2CO_3 and 120 grams of NaCl. After complete dissolution of the salts, mPEG-NH_2 was extracted into two 500 ml portions of methylene chloride. The methylene chloride layer was dried over sodium sulfate and then evaporated to dryness. The product was recrystallized from 1L of isopropanol. The product was characterized by non-aqueous titration according to the method described in Eur. J. Biochem. 42, 151 (1974) and by ^{1} NMR and ^{13}C NMR.

EXAMPLE 2

Preparation of mPEG-NCO

In this Example, 50 grams (10 mmol) of mPEG-NH_2 was placed in a 500 ml flask and dried by azeotropic distillation with toluene. The flask was cooled to 65-70°C and 1.5 grams, (5 mmol), of bis-(trichloromethyl)carbonate (triphosgene) and 2 grams, (20 mmol), of triethylamine were added and stirred at this temperature for 2 hours before dry nitrogen was bubbled through the reaction mixture for 30 minutes. The mixture was then filtered at 40°C to remove triethylamine hydrochloride. The filtrate was evaporated to near

dryness and precipitated with 250 milliliters of ether. The precipitate was dried under high vacuum. The product showed 100% activity by non-aqueous titration. The FTIR showed a strong peak at 2263 cm^{-1} and ^{13}C NMR gave a peak at 43.08 ppm for CH_2NCO and a weak peak at 121 ppm for N=C=O.

EXAMPLE 3

m-PEG-Isothiocyanate

To a solution of 50 g (0.01mol) of m-PEG-amine hydrochloride, in 500 milliliters of chloroform at room temperature was added 2.6 g (0.023mol) of thiophosgene and 6.1 g (0.06mol) of triethylamine. The resulting mixture was then refluxed for 2 hours, followed by removal of the solvent by distillation *in vacuo* to yield a semi-solid residue. This residue was recrystallized from 2-propanol to yield 45.6 g of the product (90% yield). FT-NMR ^{13}C assignments: OCH_3, 58.51 ppm; $\underline{CH_2}NCS$, 44.81. The purity of the sample was determined by non-aqueous titrations, as in the case of PEG-isocyanate, and was found to be greater than 90% pure. The FTIR showed a strong absorption at 2111.7 cm^{-1}.

EXAMPLE 4

PEGylation of 2′-acetyltaxol using mPEG-NCO: The 7-urethane derivative

In this Example, mPEG-NCO was prepared in situ by placing 515 mg (0.010 mmol) of mPEG-NH_2 in a 100 ml round bottom flask and undergoing drying by azeotropic toluene distillation and converted to mPEG-NCO as described above. The FTIR showed the isocyanate peak at 2263 cm^{-1} of intensity similar to that obtained above (relative to polyethylene glycol peaks). The reaction mixture was cooled to room temperature and to it were added 60 mg of 2′-acetyltaxol prepared as described in Biochem. Biophys. R.S. Commun. 124, 329-336 (1984), which is incorporated by reference herein, and 10 mg Sn(II) octoate and the mixture was stirred at room temperature. The reaction was followed by HPLC on a C_8 column with 3:1 methanol-H_2O as eluent. The reaction appeared to be complete when about 75% of 2′-acetyltaxol was converted to the corresponding PEG-derivative. The reaction product was evaporated to near dryness and precipitated with ether. Most of the unreacted 2′-acetyltaxol and any 2′, 7-diacetyltaxol present remained in the ether phase. The ether was decanted, and the precipitate was recrystallized from 20 milliliters of 2-propanol. The 7-PEG urethane derivative was isolated by centrifuging,

washing with two 20 milliliter portions of 2-propanol, and finally drying under high vacuum to obtain 508 mg of purer product containing less than 1% 2'-acetyltaxol and some non-functionalized PEG.

The 7-PEG urethane derivative could be further purified by HPLC using a semipreparative C_8-column. The FTIR spectrum of the purified compound had all the characteristic peaks of PEG in addition to peaks at 1748.6, 1741.2, 1726.5, 1663 cm^{-1} which are characteristic of the 2'-acetyltaxol molecule.

EXAMPLES 5 - 6

In these Examples, conjugation of the activated PEG-NCO to the protein bovine hemoglobin (Hb), obtained from California Biological and Protein Corp. of Huntington Beach, CA were carried out. The reaction conditions included maintaining a constant pH of 7.8 and temperature of 8°C. The hemoglobin was reacted in a solution having a concentration of about 10.6%. PEG-NCO was added as a solid.

EXAMPLE 5

Hb-PEG Conjugation using 12x molar excess PEG-NCO

Hb (10.6%, 2ml) was diluted with 2 ml of pH 7.8

buffer in a jacketed reaction vessel kept at 8°C. PEG-NCO (217.6 mg, 1.08 x 10^{-5}M) was added to the reaction vessel containing the Hb. The pH was adjusted to approximately 7.8 with 1.0N NaOH. The reaction mixture was stirred uniformly and slowly for two hours at 8°C maintaining pH 7.8. Thereafter, 21 mg cysteine HCl (0.03M) and glycine solution (3.24 mg, 19µl of stock 336/2ml of pH 7.0 phosphate buffer) which is equimolar with PEG-NCO were added, and the pH was re-adjusted to 7.8 with 1N NaOH. The yield of the desired PEG-Hb was determined by HPLC retention time to be in excess of 73%

EXAMPLE 6

Hb conjugation using 18x molar excess PEG-NCO

Hb (10.6%, 3ml) was diluted with 3 ml of pH 7.8 buffer in a jacketed reaction vessel kept at 8°C. The pH was adjusted to approximately 7.8 with 1.0N NaOH. PEG-NCO (491.7 mg, 1.62x10^{-2}M) was added to the reaction vessel kept at 8°C with Hb. The reaction mixture was stirred uniformly and slowly for two hours at 8°C maintaining pH 7.8. Thereafter, 31.6 mg cysteine HCl (0.03M) and glycine solution (12.18 mg, 43.5µl of stock 336/2ml of pH 7.0 phosphate buffer) which is equimolar with PEG-NCO were added. The pH was adjusted to 7.8 with 1N NaOH and allowed to stir for five minutes. The sample

was analyzed by HPLC to determine the yield of desired PEG-Hb to be in excess of 83 %.

While there have been described what are presently believed to be the preferred embodiments of the invention, those skilled in the art will realize that changes and modifications may be made thereto without departing from the spirit of the invention and it is intended to claim all such changes and modifications as fall within the true scope of the invention.

WHAT IS CLAIMED IS:

1. A composition comprising the formula:

(III) $Z'-(O-R_1)_a-(O-R_2)_b-(O-R_3)_c-NH-CO-W-R''$

wherein R_1, R_2 and R_3 are alkyl groups;
a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b and c is between 10 and 1000;
W = O or NR, wherein R = H or an alkyl;
R" is a bio-effecting substance; and
Z' is an alkyl, cycloalkyl, branched alkyl, arylalkyl or -NH-CO-W-R"

2. The composition of Claim 1, wherein said bio-effecting substance is a polypeptide.

3. The composition of Claim 1, wherein said bio-effecting substance is a chemotherapeutic agent.

4. The composition of Claim 2, wherein said polypeptide is an enzyme.

5. The composition of Claim 4, wherein said enzyme is selected from the group consisting of asparaginase, arginase, adenosine deaminase, superoxide dismutase, catalase, chymotrypsin, lipase, uricase, bilirubin oxidase, glucose oxidase, glucuronidase, galactosidase, glucocerebrosidase and glucuronidase.

6. The composition of Claim 2, wherein said polypeptide is selected from the group consisting of Factor VIII, insulin, ACTH, glucagon, somatostatin, somatotropins, thymosin, parathyroid hormone, pigmentary hormones, somatomedins, erythropoietin, luteinizing hormone, hypothalamic releasing factors, antidiuretic hormones, prolactin, interleukins, interferons and colony

stimulating factors, hemoglobin, cytokines and antibodies.

7. The composition of Claim 2, wherein said polypeptide is a glycopolypeptide.

8. The composition of Claim 7, wherein said glycopolypeptide is selected from the group consisting of immunoglobulins, ovalbumin, lipase, glucocerebrosidase, lectins, tissue plasminogen activator and glycosilated interleukins, interferons and colony stimulating factors.

9. The composition of Claim 8, wherein said immunoglobulin is selected from the group consisting of IgG, IgE, IgM, IgA, IgD and fragments thereof.

10. The composition of Claim 3, wherein said chemotherapeutic agent is taxol or a taxol derivative.

11. The composition of Claim 3, wherein said chemotherapeutic agent is selected from the group consisting of anti-infectives, anti-inflammatories, anti-tumor agents, analgesics, cardiac agents, steroids and central nervous system agents.

12. A method of preparing a composition of Claim 1, comprising reacting a first composition corresponding to the formula:

(I) $Z - (O - R_1)_a - (O - R_2)_b - (O - R_3)_c - Y$

wherein:

R_1, R_2 and R_3 are alkyl groups;

a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b and c is between 10 and 1000;

Y is NCX, wherein X is one of O or S; and

Z is one of an alkyl, cycloalkyl, branched alkyl, arylalkyl group or Y.

with a bio-effecting substance under conditions sufficient to effect conjugation of said composition and said bio-effecting molecule.

13. The method of Claim 12, wherein said conditions include reacting said first composition with said bio-effecting substance in a molar ratio of from about 0.1 : 1 to about 50 : 1.

14. The method of Claim 13, wherein said conditions include reacting said first composition with said bio-effecting substance in a molar ratio of from about 1 : 1 to about 25 : 1.

15. The method of Claim 14, wherein said conditions include reacting said composition of Claim 1 with said bio-effecting substance in a molar ratio of from about 5 : 1 to about 20 : 1.

16. The method of Claim 12, wherein said bio-effecting substance is a polypeptide.

17. The method of Claim 16, wherein said polypeptide is an enzyme.

18. The method of Claim 12, wherein said bio-effecting molecule is a chemotherapeutic agent.

19. The method of Claim 18, wherein saidchemotherapeutic agent is taxol or a taxol derivative.

20. The method of Claim 18, wherein said chemotherapeutic agent is selected from the group consisting of anti-infectives, anti-inflammatories, anti-tumor agents, analgesics, cardiac agents, steroids and central nervous system agents.

AMENDED CLAIMS
[received by the International Bureau on 24 January 1994 (24.01.94); original claims 1-20 cancelled; original claims 21-36 amended; (3 pages)]

21. A bio-effecting conjugate, comprising a chemotherapeutic agent linked to a polyalkylene oxide via a urethane linkage.

22. The bio-effecting conjugate of claim 21, wherein said chemotherapeutic agent is taxol or a taxol derivative.

23. The bio-effecting conjugate of claim 21, wherein said chemotherapeutic is selected from the group consisting of anti-infectives, anti-inflammatories, anti-tumor agents, analgesics, cardiac agents, steroids and central nervous system agents.

24. The bio-effecting conjugate of claim 21, wherein said polyalkylene oxide comprises polyethylene glycol.

25. The bio-effecting conjugate of claim 21, wherein said polyalkylene oxide has a molecular weight of from about 600 to about 100,000 daltons.

26. The bio-effecting conjugate of claim 25, wherein said polyalkylene oxide has a molecular weight of from about 2,000 to about 20,000 daltons.

27. The bio-effecting conjugate of claim 26, wherein said polyalkylene oxide has a molecular weight of from about 3,000 to about 7,000 daltons.

28. A method of preparing a bio-effecting conjugate containing a chemotherapeutic agent linked to a polyalkylene oxide urethane linkage, comprising:

reacting an isocyanate-activated polyalkylene oxide with a chemotherapeutic agent in the presence of a tin-based catalyst.

29. The method of 28, wherein said chemotherapeutic agent is taxol or a taxol derivative.

30. The method of claim 28, wherein said chemotherapeutic is selected from the group consisting of anti-infectives, anti-inflammatories, anti-tumor agents, analgesics, cardiac agents, steroids and central nervous system agents.

31. The method of claim 28, wherein said polyalkylene oxide comprises polyethylene glycol.

32. The method of claim 28, wherein said polyalkylene oxide has a molecular weight of from about 600 to about 100,000 daltons.

33. The method of claim 28, wherein said polyalkylene oxide has a molecular weight of from about 2,000 to about 20,000 daltons.

34. The method of claim 28, wherein said polyalkylene oxide has a molecular weight of from about 3,000 to about 7,000 daltons.

35. The method of claim 28, wherein said tin-based catalyst is dibutyltin dilaurate or Sn(II) Octoate.

36. The method of claim 28, wherein further comprising conducting said reacting in a non-aqueous environment.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07579

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 47/48; C07C 265/14,221/30
US CL :435/188; 558/17,18; 560/347,357
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/188; 558/17,18; 560/347,357

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE - STRUCTURE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	EP, A, 0,510,356 (Hakimi et al) 28 October 1992, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
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